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Mechanisms of Extracellularly Regulated Kinases 1/2 Activation in Adrenal Glomerulosa Cells by Lysophosphatidic Acid and Epidermal Growth Factor

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The regulation of adrenal function, including aldosterone production from adrenal glomerulosa cells, is dependent on a variety of G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). In many cell types, GPCR-mediated MAPK activation is mediated through transactivation of RTKs, in particular the epidermal growth factor (EGF) receptor (EGF-R). However, the extent to which this cross-communication between GPCRs and RTKs is operative in the adrenal glomerulosa has not been defined. Bovine adrenal glomerulosa cells express receptors for lysophosphatidic acid (LPA) and EGF. In cultured bovine adrenal glomerulosa cells, LPA, which is predominantly coupled to G_i and partially to G_q/protein kinase C α and ϵ, caused phosphorylation of Src (at Tyr416), proline-rich tyrosine kinase (Pyk2 at Tyr402), EGF-R, protein kinase B/Akt, extracellularly regulated signal kinases 1/2, and their dependent protein, p90 ribosomal S6 kinase. Overexpression of dominant negative mutants of Ras or EGF-R, and selective inhibition of EGF-R kinase with AG1478, significantly reduced LPA-induced ERK1/2 phosphorylation. However, this was not impaired by inhibition of matrix metalloproteinase (MMP) and heparin-binding EGF. LPA-induced ERK1/2 activation occurs predominantly through EGF-R transactivation by G_i/Src and partly through activation of protein kinase C, which acts downstream of EGF-R and Ras. In contrast, LPA-induced phosphorylation of Shc and ERK1/2 in clonal hepatocytes (C9 cells) was primarily mediated through MMP-dependent transactivation of the EGF-R. These observations in adrenal glomerulosa and hepatic cells demonstrate that LPA phosphorylates ERK1/2 through EGF-R transactivation in a MMP-dependent or -independent manner in individual target cells. This reflects the ability of GPCRs expressed in cell lines and neoplastic cells to utilize distinct signaling pathways that can elicit altered responses compared with those of native tissues. (*Molecular Endocrinology* 19: 2535–2548, 2005)

ADRENAL ZONA GLOMERULOSA cells synthesize and secrete aldosterone, an essential regulator of sodium/potassium and fluid balance and acid-base homeostasis. Recently, aldosterone has been found to also exert deleterious actions on cardiovascular function (1, 2). Aldosterone secretion by glomerulosa cells is stimulated by extracellular K^+, angiotensin II, corticotropin, and other G protein-coupled receptor (GPCR) and receptor tyrosine kinase (RTK) agonists. The growth and proliferation of glomerulosa cells are regulated by GPCRs and RTKs, and activation of MAPKs (3). The role of GPCRs in the modulation of aldosterone synthesis has been extensively studied, and RTK activation by epidermal growth factor (EGF) has been shown to enhance the stimulatory actions of GPCRs (4, 5). However, little information is available on the signaling pathways involved in MAPK activation by GPCRs and RTKs in adrenal glomerulosa cells. Moreover, the extent to which cross-communication between GPCRs and RTKs occurs in these cells has not been established (6).

MAPKs are involved in cell survival, growth, secretion, chemotaxis, and motility. Cells utilize a wide variety of pathways in transducing signals from plasma membrane receptors to MAPKs, especially ERK1/2. Recent studies have revealed that activation of MAPK by external stimuli, such as GPCR agonists, cytokines, growth hormones, steroids, and environmental stresses, often occurs through transactivation of RTKs, in particular the EGF receptor (EGF-R). Activation and tyrosine phosphorylation of RTKs leads to recruitment of specific signaling proteins and adaptor molecules, culminating in sequential activation of the

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Abbreviations: BAG, Bovine adrenal granulosa; CRM, CRM197 diphtheria toxin mutant; P-ERK1/2, phosphorylated ERY1/2; dn, dominant negative; EGF, epidermal growth factor; EGF-R, EGF receptor; GPCR, G protein-coupled receptor; HB-EGF, heparin binding EGF; LPA, lysophosphatidic acid; MEK, MAPK kinase; MMP, matrix metalloproteinase; P-ERK1/2, phosphorylated ERK1/2; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; Pyk2, proline-rich tyrosine kinase; RSK-1, p90 ribosomal S6 kinase 1; RTK, receptor tyrosine kinase.

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Ras/Raf/MAPK kinase (MEK)/ERK cascade (7–10). Although the mechanism of ERK1/2 activation by RTKs is well defined, the steps leading from GPCR stimulation to RTK tyrosine phosphorylation are only partially understood (7).

Lysophosphatidic acid (LPA) is a naturally occurring lysophospholipid with a wide range of biological actions, particularly as an inducer of cell proliferation, migration, and survival. LPA is also involved in wound healing, brain development, vascular remodeling and platelet aggregation, and tumor progression (11–14), and regulates Ca^{2+} efflux from adrenal chromaffin cells through activation of protein kinases (15). Although LPA receptors are predominantly coupled to G_i, they also interact with G_q and G_s in a cell type-specific manner (12, 13). LPA receptors are expressed in bovine adrenal glomerulosa (BAG) cells, but the signaling pathways activated by LPA and their significance in glomerulosa cell function have not been investigated. An examination of the mechanism of LPA-induced ERK1/2 activation and cell proliferation in BAG cells showed that LPA is predominantly coupled to G_i and causes phosphorylation of ERK1/2 (P-ERK1/2) and p90 ribosomal S6 kinase 1 (RSK-1) through transactivation of the EGF-R in a metalloproteinase-independent manner. Furthermore, LPA caused proliferation of BAG cells through activation of Src and phosphoinositide 3-kinase (PI3K). In contrast, LPA-induced phosphorylation of Shc, ERK1/2, and RSK-1 in C9 cells was primarily mediated through matrix metalloproteinase (MMP)-dependent transactivation of the EGF-R. These findings indicate that LPA elicits mitogenic effects through EGF-R transactivation in a metalloproteinase-dependent or -independent manner in specific cell types.

RESULTS

Agonist activation of LPA and EGF receptors in BAG cells caused rapid phosphorylation of ERK1/2, with a maximum effect at 5 min and a decline after 15 min (Fig. 1). GPCR-mediated ERK1/2 activation frequently involves the generation of signals that lead to transactivation and tyrosine phosphorylation of the EGF-R. In many cell types, the growth-promoting effects of GPCRs are primarily mediated by transactivation of the EGF-R, with subsequent activation of signaling pathways such as the Ras/Raf/MEK/ERK/RSK cascade and PI3K/Akt (10, 16). Both LPA and EGF cause tyrosine phosphorylation of the EGF-R, as revealed by immunoprecipitation of cell lysates of BAG cells with anti-EGF-R antibody and immunoblotting with phosphotyrosine antibody (Fig. 2A). Treatment of cells with the selective EGF-R kinase antagonist, AG1478, abolished EGF-induced ERK1/2 activation and significantly attenuated the LPA-induced response (Fig. 2, B and C). Similarly, inhibition of EGF-R activation by overexpression of a dominant negative (dn) EGF-R mutant abolished EGF-induced ERK1/2 phosphorylation and significantly attenuated LPA responses (Fig. 2D). These findings indicate that LPA exerts its stimulatory effects on ERK1/2 phosphorylation in BAG cells largely through transactivation of the EGF-R. The effects of specific inhibitors showed that LPA-induced tyrosine phosphorylation of the EGF-R is also dependent on activation of G_i and Src, but is independent of protein kinase C (PKC) and metalloproteinases (Fig. 2E).

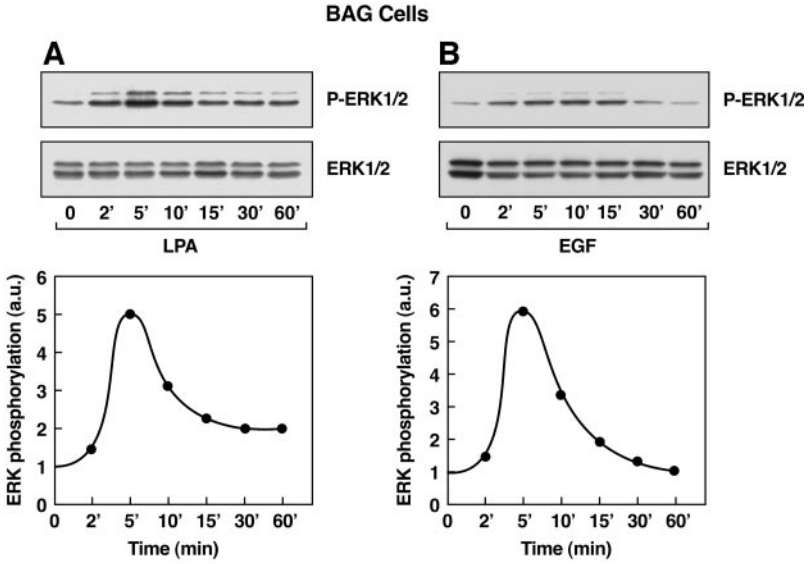

Fig. 1. LPA and EGF Cause Transient Activation of ERK1/2

A, Time course of the effects of LPA and EGF on phosphorylation of ERK1/2. Serum-starved BAG cells were treated with LPA (10 μM) and EGF (20 ng/ml) for the time periods indicated, lysed in Laemmli sample buffer, and analyzed by SDS-PAGE using phospho-specific antibodies against ERK1/2 (Thr202/Tyr204). The blots were stripped and reprobed with ERK1/2 antibodies. ERK1/2 phosphorylation in unstimulated cells at time zero min (control) was taken as 1, and agonist-induced increases in phosphorylation were compared with control as arbitrary units (a.u.). The data shown are representative of three experiments.

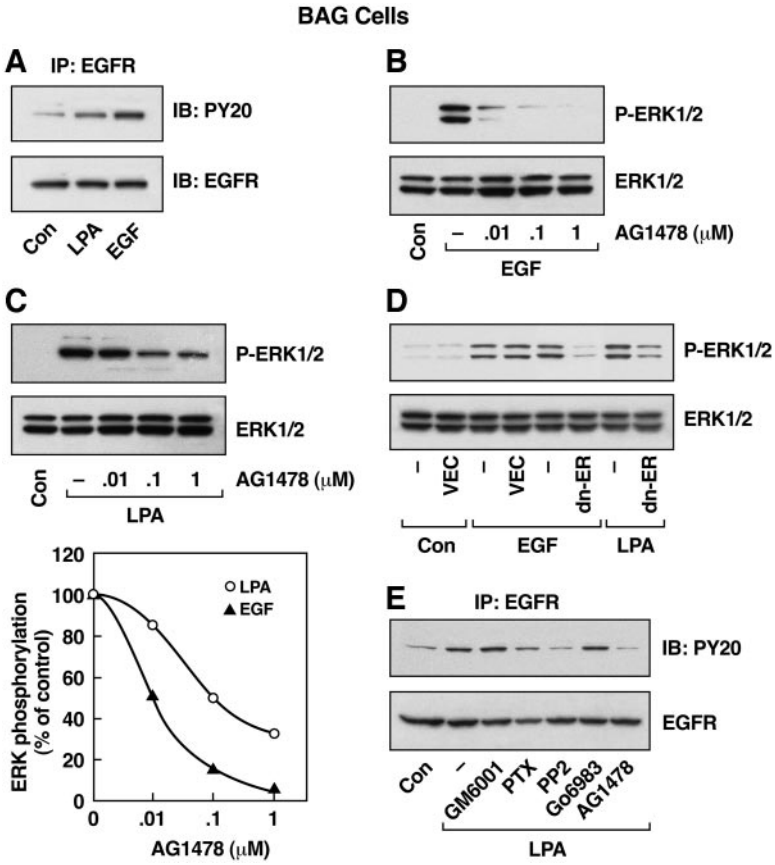

Fig. 2. Dependence of LPA-Induced ERK1/2 Phosphorylation on EGF-R Transactivation, but Not Metalloproteinase Activation, in BAG Cells

A, BAG cells were treated with LPA and EGF for 2 min, after which cell lysates were immunoprecipitated with EGF-R antibody and analyzed by immunoblotting with phosphotyrosine antibody (PY20). B and C, Concentration-dependent inhibitory effects of EGF-R kinase inhibition on ERK1/2 phosphorylation by EGF and LPA. Cells were treated with increasing concentrations of AG1478 for 20 min before stimulation with LPA (10 μM) and EGF (20 ng/ml) for 5 min. D, Overexpression of dominant negative EGF-R plasmid cDNA (dnER: 1 μg), but not the vector plasmid DNA (VEC), impairs agonist-induced ERK1/2 activation. Cells were transfected with cDNA as described in *Materials and Methods*. E, LPA-induced phosphorylation of the EGF-R is dependent on G_i and Src, but independent of MMPs and PKC. Cells were treated with PTX (50 ng/ml for 16 h), MMP inhibitor, GM6001 (20 μM), Src inhibitor, PP2 (10 μM), PKC inhibitor, Go6983 (1 μM), and EGF-R antagonist, AG1478 (100 nM) for 30 min, and stimulated with LPA (10 μM) for 4 min. Cell lysates were analyzed by immunoprecipitating EGF-R and immunoblotting with phosphotyrosine antibody (PY20). Con, Control; IB, immunoblot; IP, immunoprecipitation.

One of the major mechanisms involved in the GPCR-mediated transactivation of the EGF-R is MMP-dependent release of heparin-binding EGF (HB-EGF), which binds to and activates the EGF-R, culminating in activation of the ERK1/2 cascade (10, 16, 17). However, blockade of MMP action by the broad-spectrum inhibitor, GM6001, which prevents LPA-induced ERK1/2 activation in rat-1 cells (18), had no significant inhibitory effect on agonist-induced ERK1/2 responses (Fig. 3, A and B), thus excluding a role of MMP in transactivation of the EGF-R and subsequent ERK1/2 phosphorylation in BAG cells. Furthermore, the selective HB-EGF antagonist, CRM, and a neutralizing antibody against HB-EGF, also had no effects on ERK/12 phosphorylation by LPA (Fig. 3C), indicating that HB-EGF generation through MMP induction is not involved in this cascade.

We next determined the upstream signaling molecules involved in agonist-induced ERK1/2 activation. Although LPA receptors are mainly coupled to G_i, their interaction with G_q has also been reported (12, 13). Blockade of G_i with pertussis toxin (PTX; 100 ng/ml for 16 h) abolished LPA-induced activation of both ERK1/2 and its dependent protein, RSK-1 (Fig. 4A). Furthermore, PKC depletion by overnight treatment with phorbol myristate acetate (PMA; 1 μM) caused a partial decrease (40%) in LPA responses. Consistent with the intermediary role of PKC, stimulation of BAG cells with PMA (200 nM) caused marked phosphorylation of ERK1/2 and RSK-1, which was abolished by PKC depletion but not by inhibition of G_i with PTX (Fig. 4B). However, EGF-induced activation of ERK1/2 and RSK-1 was significantly decreased by PTX treatment, but not by PKC depletion (Fig. 4C). LPA has been

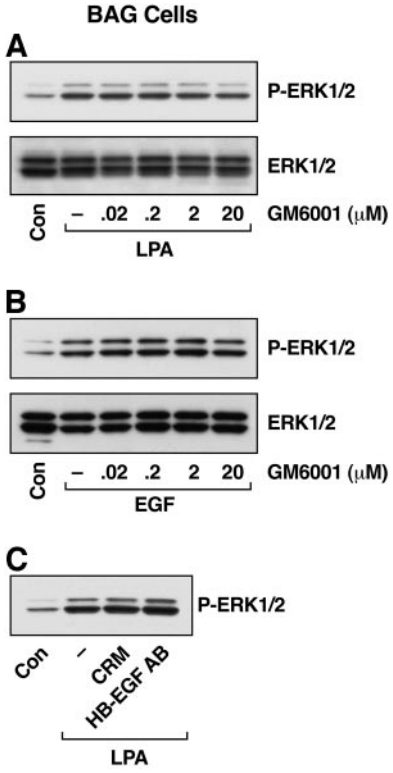

Fig. 3. Lack of Metalloproteinase Involvement in Agonist-Induced ERK1/2 Activation in BAG Cells

A and B, Cells were treated with increasing concentrations of metalloproteinase inhibitor, GM6001, for 20 min and stimulated with LPA (10 μM) and EGF (20 ng/ml) for 5 min. C, Lack of effects of HB-EGF antagonist, CRM (10 μM), and anti-HB-EGF antibody (10 μg/ml) on LPA-induced ERK1/2 activation. Con, Control.

shown to cause activation of PKC in rat-1 cells (11, 19). Inhibition of PKC by Go6983 (1 μM) reduced the ERK1/2 activation induced by LPA and PMA, but not by EGF (Fig. 4D). Moreover, LPA-induced ERK1/2 activation is decreased by overexpression of dnPKCα and -ϵ, but not by PKCδ (Fig. 4E). These data indicate that G_i activation is critical in transducing signals from both LPA and EGF, and that PKC activation is involved only in LPA signaling in BAG cells.

Important roles of nonreceptor tyrosine kinases such as Src and proline-rich tyrosine kinase (Pyk2) have been reported during GPCR-mediated EGF-R transactivation in various cell types (20, 21). An examination of the roles of these signaling proteins in BAG cells revealed that LPA caused phosphorylation of Src at Tyr416 and Pyk2 at Tyr 402. Furthermore, the selective Src inhibitor, PP2, attenuated the stimulatory effects of LPA on phosphorylation of Src and Pyk2 as well as ERK1/2 (Fig. 5A). EGF-induced ERK responses were also abolished by PP2 in a concentration-dependent manner (Fig. 5B). Overexpression of C-terminal Src kinase, which acts a negative regulator of Src kinase (22), also reduced agonist-induced responses in BAG cells (Fig. 5C), confirming the critical role of Src in BAG cell signaling.

EGF elicits ERK1/2 activation through recruitment of adaptor molecules such as Shc, Grb, and Sos, which cause activation of the Ras, Raf, and MEK/ERK1/2 cascade (9). To determine whether LPA-induced responses following EGF-R transactivation are mediated through activation of Ras, we measured ERK1/2 activation in BAG cells overexpressing dnRas (S17N). As shown in Fig. 6A, dnRas markedly reduces LPA- and EGF-stimulated phosphorylation of ERK1/2, indicating that LPA mediates its effects in BAG cells largely through an EGF-R- and Ras-dependent pathway. An analysis of the mechanism of Ras activation (Fig. 6B) indicates that LPA-induced Ras activation is independent of MMP and PKC activation but is dependent on G_i and EGF-R activation. This suggests that the partial involvement of PKC in LPA-induced ERK1/2 phosphorylation is independent of EGF-R and Ras activation in BAG cells. Consistent with this, whereas blockade of EGF-R kinase by AG1478 (100 nM) and PKC by Go6983 (1 μM) caused partial inhibition of ERK1/2 activation by LPA, their simultaneous blockade completely abolished LPA responses in BAG cells (Fig. 6C), indicating that LPA exerts its effects on ERK1/2 through two distinct signaling cascades in BAG cells.

It is clear that LPA-induced ERK1/2 phosphorylation in native BAG cells is partly dependent on EGF-R transactivation, but is independent of MMP induction (Fig. 2). To determine the role of MMP involvement in LPA-induced activation of EGF-R and ERK1/2 in another cell type, we used clone 9 hepatocyte cell line (C9 cells), which also expresses endogenous receptors for LPA and EGF (20). In these cells, both agonists caused transient phosphorylation of ERK1/2 (Fig. 7, A and B), and the response to LPA, but not EGF, was mediated solely by the pertussis toxin-sensitive G_i cascade (Fig. 7C). Our data in BAG cells showed that PKC has a partial role in LPA-induced ERK1/2 activation. When we examined the role of PKC in LPA signaling in C9 cells, the PKC inhibitor, Go6983, abolished the effects of PMA but had no effects on ERK1/2 activation by LPA and EGF, thus excluding a role of PKC in LPA signaling in C9 cells (Fig. 7D).

LPA and EGF stimulation of C9 cells caused rapid tyrosine phosphorylation of the EGF-R as determined by its immunoprecipitation and immunoblotting with phosphotyrosine antibody (Fig. 8A). Moreover, pretreatment of cells with the EGF-R kinase antagonist, AG1478, completely abolished Akt and ERK1/2 activation by LPA and EGF (Fig. 8, B and C), indicating the critical role of EGF-R transactivation in LPA-induced responses in C9 cells. However, unlike its action in BAG cells, MMP inhibition by GM6001 significantly attenuated activation of ERK1/2 by LPA (Fig. 8D). These data suggest that LPA-induced EGF-R transactivation is dependent on MMP activation in C9 cells. It is well established that agonist-induced MMP induction causes generation of HB-EGF, which by phosphorylating the EGF-R causes activation of Shc/Grb/

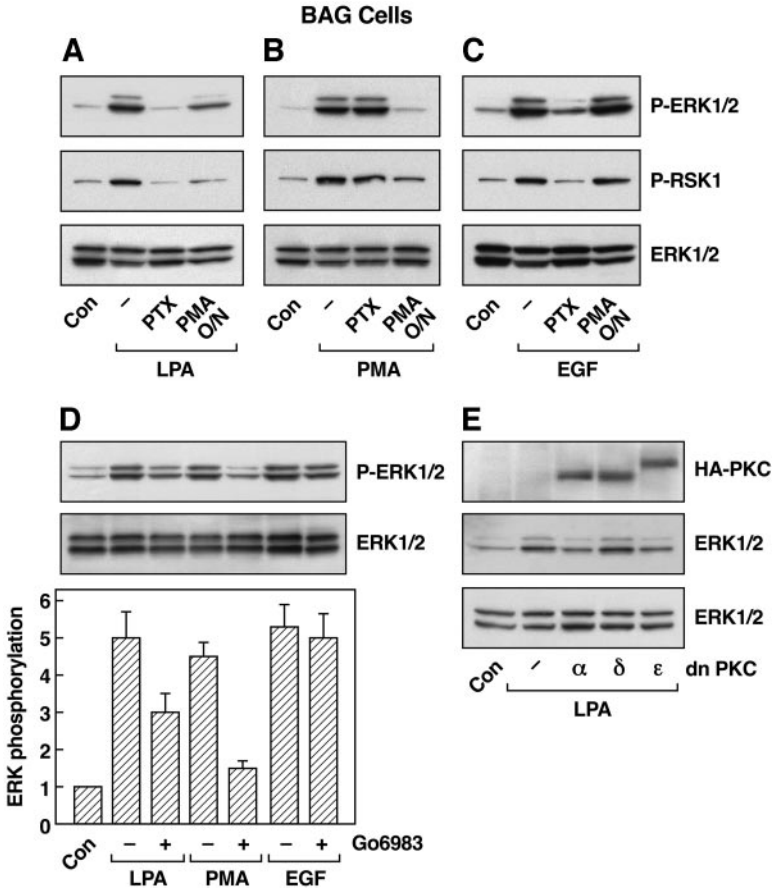

Fig. 4. Roles of G_i and PKC in Agonist-Induced Phosphorylation of ERK1/2 and RSK-1 in BAG Cells

A–C, Cells were treated with PTX (100 ng/ml) for 16 h and PMA (1 μM) overnight (O/N) followed by stimulation with LPA (10 μM), PMA (100 nM), and EGF (20 ng/ml) for 5 min. D, Cells were treated PKC inhibitor, Go6983 (1 μM), for 20 min followed by stimulation with LPA, PMA, and EGF. E, Effects of overexpression of dominant negative (dn) PKCα, -δ, and -ϵ on LPA-induced ERK1/2 activation. BAG cells were transfected as described in *Materials and Methods*. The expression of dnPKCs was confirmed by immunoblotting with hemagglutinin (HA)-tag antibody indicated as HA-PKC (*upper panel*). Con, Control; P-RSK1, phosphorylated RSK-1.

Sos and the MEK/ERK cascade (10). Consistent with this, MMP inhibition by GM6001 markedly attenuated LPA-induced phosphorylation of Shc in C9 cells (Fig. 8E).

To determine whether HB-EGF causes ERK1/2 activation through phosphorylation of the EGF-R, we examined the signaling pathways activated by HB-EGF. Treatment of C9 cells with HB-EGF caused significant but transient phosphorylation of EGF-R (Y1173), Shc, and ERK1/2 (Fig. 9A). Phosphorylation of the EGF-R and Shc by HB-EGF was abolished by the selective EGF-R kinase antagonist, AG1478, but not by the selective inhibition of MEK by PD98059, metalloproteinase(s) by GM6001, PKC by Go6983, and Src by PP2. However, reprobing the same blot for phospho-ERK1/2 revealed that its phosphorylation by HB-EGF was dependent on activation of the EGF-R and MEK1/2 (Fig. 9B). These data demonstrate that the effects of HB-EGF are mediated by activation of the EGF-R, and that HB-EGF release is responsible for LPA-induced transactivation of the EGF-R in C9 cells.

These studies have shown that both LPA and EGF cause activation of $G_{i,}$ which is known to mediate downstream signals through activation of PI3K in specific cell types (11, 23, 24). The extent to which LPA utilizes the PI3K/Akt cascade in these cells is not known. Time-course studies revealed that LPA causes phosphorylation of Akt at Ser473 as early as 2 min, and the PI3K-dependence of this effect was indicated by its abolition by wortmannin in both C9 and BAG cells (Fig. 10, A and B, *top panel*). Whereas treatment of BAG cells with varying concentrations of wortmannin caused a decrease in ERK1/2 activation by LPA in BAG cells, it had no effects in C9 cells (Fig. 10, A and B, *lower panel*). Because LPA is well known to exert mitogenic effects in various cell types (12), and LPA-induced transactivation of the EGF-R and DNA synthesis were prevented by the MMP inhibitor, GM6001, in rat-1 cells (18), we also determined the effects of LPA stimulation on cell proliferation. LPA-induced proliferation of C9 cells (50%) was greater than in BAG cells (25–30%). Individual inhibition of Src, EGF-R, PI3K, and

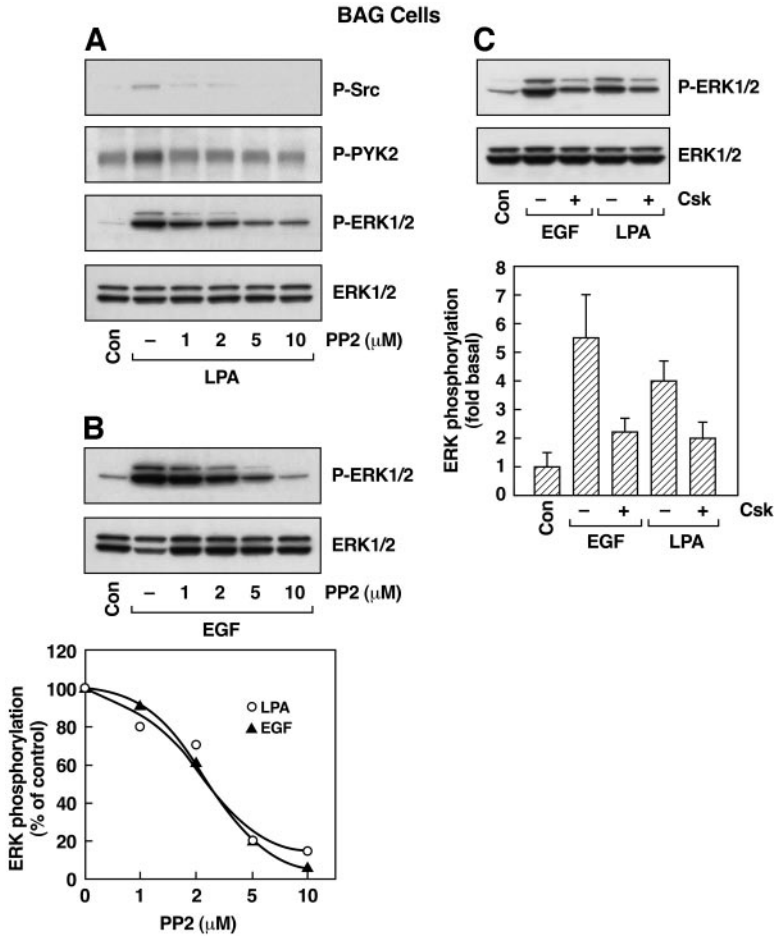

Fig. 5. Role of Src in Agonist-Induced ERK1/2 Activation in BAG Cells

A, Effects of Src inhibition by PP2 on LPA-induced phosphorylation of Src (Y416), Pyk2 (Y402), and ERK1/2. BAG cells were treated with increasing concentrations of PP2 for 20 min and stimulated with LPA (10 μM for 5 min). B, Effects of Src inhibition on ERK1/2 activation induced by EGF (20 ng/ml). C, Effects of overexpression of negative regulatory Src kinase (Csk; 1 μg) on ERK1/2 activation by EGF and LPA. Cells were lysed in Laemmli sample buffer and analyzed by SDS-PAGE. The quantitation of data is shown as mean ± SEM (n = 3). Con, Control; P-Src, phosphorylated Src; P-PYK2, phosphorylated Pyk2.

MEK caused a decrease of LPA-induced responses in both cell types. However, inhibition of MMP attenuated proliferation only in C9 cells (Fig. 10, C and D), consistent with a role of MMP in LPA-induced EGF-R transactivation and ERK1/2 phosphorylation in these cells. Conversely, PKC inhibition reduced LPA-induced proliferation in BAG cells, but not in C9 cells (Fig. 10, C and D). These data indicate that LPA utilizes differential signaling pathways during ERK1/2 activation and proliferation in BAG and C9 cells.

DISCUSSION

Although the cross-communication between GPCRs and RTKs (*e.g*. EGF-R) during GPCR agonist-induced ERK1/2 activation has been demonstrated in numerous cell lines and tumor cells, relatively little information is available about primary cultures of native endocrine cells. BAG cells, which express several GPCRs (including those for LPA, angiotensin II, bradykinin, and endothelin-1) and RTKs for EGF and fibroblast growth factor (3, 6, 21), provide a physiological model for investigation of the mechanism(s) of cross-communication between these receptors during ERK1/2 activation. The present study has yielded three significant observations. First, that LPA and EGF share the same signaling pathways to ERK1/2 phosphorylation, in terms of their dependence on activation of G_i and Src. Second, LPA-induced ERK1/2 phosphorylation is partly dependent on transactivation of the EGF-R in BAG cells but is completely dependent in C9 cells. In BAG cells, LPA causes ERK1/2 activation through both EGF-R- and PKC-dependent cascades. Third, LPA-induced EGF-R transactivation is mediated through MMP-dependent HB-EGF generation in C9 cells, but not in BAG cells.

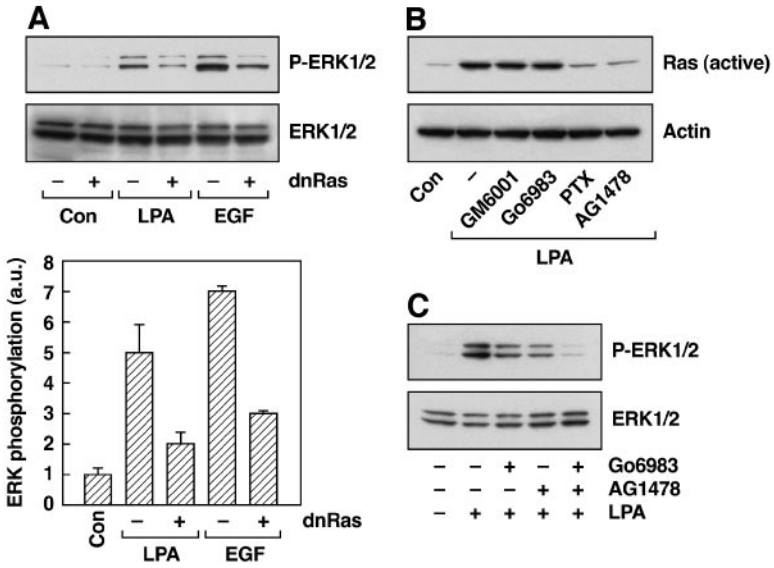

Fig. 6. LPA Signaling Occurs through Ras Activation in BAG Cells

A, Overexpression of dominant negative Ras (dnRas; S17N) plasmid cDNA (1 μg) impairs agonist-induced ERK1/2 activation. Cells were transfected with cDNA as described in *Materials and Methods*. B, BAG cells were treated with PTX (100 ng/ml) for 16 h, and GM6001 (20 μM), Go6983 (1 μM), and AG1478 (100 nM) for 20 min followed by stimulation with LPA (10 μM) for 5 min. Agonist-stimulated Ras activation was measured as described in *Materials and Methods*. C, Effects of coaddition of AG1478 (100 nM) and Go6983 (1 μM) on LPA-induced ERK1/2 activation. Con, Control.

LPA causes proliferation, migration, and mitogenic responses including MAPK phosphorylation through EGF-R transactivation in various cell types (11, 25, 26). One of the major mechanisms mediating cross-communication between GPCRs and RTKs is the activation of MMPs causing the release of several ligands such as HB-EGF, amphiregulins, TGF-α, and TNF-α, which bind to and activate the EGF-R (10, 16, 17, 22, 27–30). The ADAM family of MMPs has been implicated in the transactivation of EGF-R and cell proliferation by GPCR agonists such as angiotensin II, endothelin-1, phenylepherine, bombesin, and LPA (25, 30–40). Much of this work has been performed in cancer cells and cell lines, and the sparsity of information in primary native cells raises a question about the physiological relevance of these observations.

Our data show that whereas LPA-induced ERK1/2 phosphorylation is markedly decreased by GM6001 in C9 hepatic cells, it is not affected in BAG cells by concentrations of GM6001 that are known to inhibit agonist-induced MMP induction (18, 30, 38), thus excluding a role of MMP in EGF-R transactivation during LPA action in these cells. In BAG cells, LPA signaling is primarily mediated by Src kinase, which is known to cause intracellular phosphorylation of the EGF-R at Tyr845 (41) and Tyr1068 (42). Interestingly, Src has been implicated in agonist-induced induction of MMPs and subsequent EGF-R transactivation in various cell types (30, 39, 43, 44). However, the reason why LPA bypasses MMP activation during EGF-R activation in BAG cells is not clear. Previous studies have shown that the expression and activation of signaling molecules is altered in cancer cells. For example, MMP levels are low in normal liver, but many fold higher in hepatocellular carcinomas (45). Similarly, PI3K/Akt activity is high in H295R cells derived from a human adrenal cortical carcinoma, but low in primary cultures of BAG cells (46). Furthermore, MAPK activation by angiotensin II is dependent on MMP-mediated transactivation of the EGF-R in clonal C9 hepatic cells (30), but is independent of MMP/EGF-R activation in native rat hepatocytes (47, 48). Thus, the signaling pathways in cell lines and cancer cells may often be altered and do not necessarily reflect the biochemical mechanisms of native cells.

Whereas substantial data point to the importance of EGF-R activation in GPCR-mediated MAPK signaling, there are relatively few reports on the role of this mechanism in native cell types (7, 10, 30, 49, 50). Our data show that the potent EGF-R antagonist, AG1478, which completely abolishes the effects of both LPA and EGF in C9 cells, cannot exceed 70% inhibition of LPA-induced ERK1/2 activation in BAG cells (Fig. 2). Similarly, the selective inhibition of Src does not completely abolish the effects of LPA in BAG cells (Fig. 5). Thus, a minor but distinct component of LPA signaling is independent of Src and EGF-R transactivation in these cells. This appears to be due to a PKC-dependent pathway, because LPA-induced ERK1/2 activation was partly attenuated by inhibition of PKC as well as in PKC-depleted cells (Fig. 4). However, PKC inhibition had no effects on LPA-induced activation of the EGF-R and Ras (Figs. 2 and 6). Moreover, simultaneous blockade of EGF-R and PKC completely abolished LPA responses in BAG cells (Fig. 6C), indicating that LPA exerts its actions through two distinct cas-

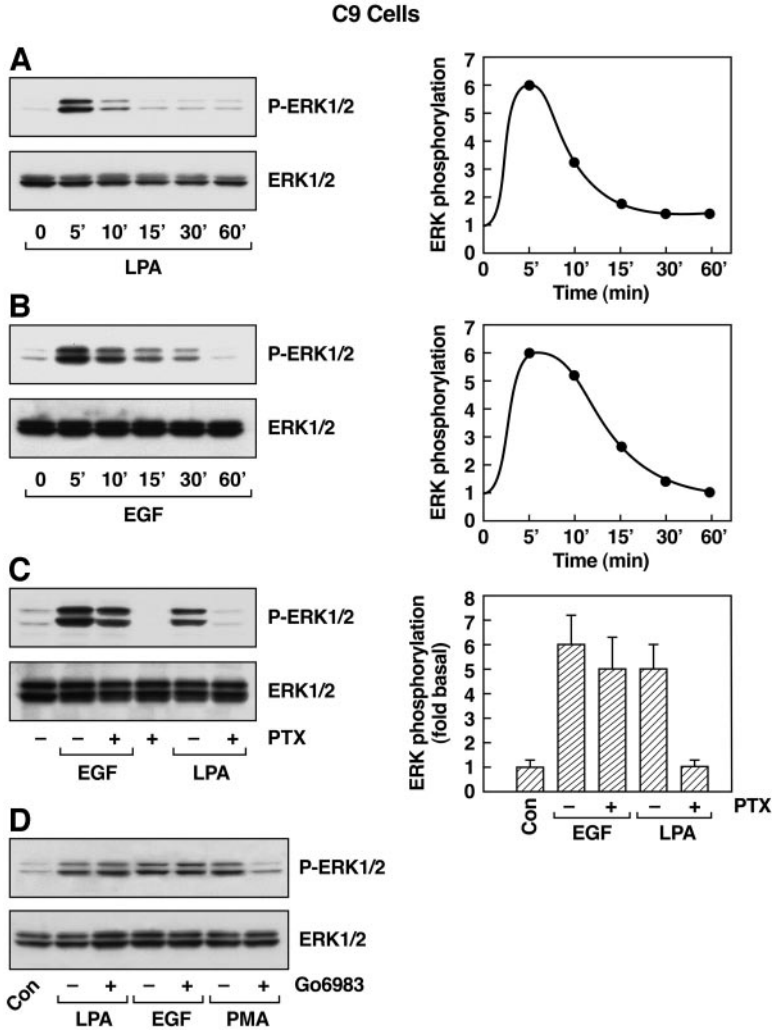

Fig. 7. Activation of ERK1/2 by LPA and EGF in Hepatic C9 Cells

A and B, Time course of LPA- and EGF-induced phosphorylation of ERK1/2 and RSK-1 in C9 cells. Cells were treated with LPA (10 μM) and EGF (20 ng/ml) for the time periods indicated. C, Effects of PTX (100 ng/ml for 16 h) on agonist-induced ERK1/2 activation. *Right panels* show the quantitation of data. D, Effects of PKC inhibition on agonist-induced ERK1/2 activation. C9 cells were treated with PKC inhibitor, Go6983 (1 μM), for 20 min and stimulated with LPA, PMA, and EGF for 5 min. Con, Control.

cades. In contrast, LPA causes its effects in C9 cells primarily through G_i and MMP-dependent EGF-R transactivation, but is independent of PKC (Figs. 7 and 8). These and other data (7, 30) indicate that the involvement of a multiplicity of signaling pathways during agonist-induced ERK1/2 activation is not uncommon. For example, ERK1/2 activation by bradykinin in COS-7 cells occurs via a dual signaling pathway involving the independent activation of PKC and transactivation of the EGF-R (51). In contrast, more recent studies showed that ERK1/2 activation in human embryonic kidney 293 cells by angiotensin II, a G_q/PKC-coupled GPCR, is independent of EGF-R transactivation (30) but is dependent on both PKC and β-arrestin2 (52). Whether LPA signals through β-arrestin2 in BAG cells has yet to be investigated.

The present results in BAG cells demonstrate that whereas a G_i-linked cascade is the predominant pathway mediating the effects of LPA on phosphorylation of Src/Pyk2 and ERK1/2, activation of PKC also contributes to LPA signaling. GPCRs coupled to G_i-linked cascades are known to cause activation of Src and PI3K through their interaction with released $\beta\gamma$-subunits (11, 23, 24). Our data in BAG cells showed that LPA causes ERK1/2 phosphorylation through G_i and Src, with the additional involvement of the EGF-R. Interestingly, EGF also exerts its effects on ERK1/2 activation through G_i and Src in BAG cells, but is independent of PKC (Figs. 4 and 5). Although this is not common, a G_i-linked cascade also mediates ERK1/2 activation by EGF in normal hepatocytes (53) and ovarian theca cells (54), and in airway smooth muscle cells after stimulation with platelet-derived growth factor (55). Thus, LPA appears to utilize a similar pathway through activation of EGF-R in exerting its stimulatory effects on MAPK signaling in BAG cells.

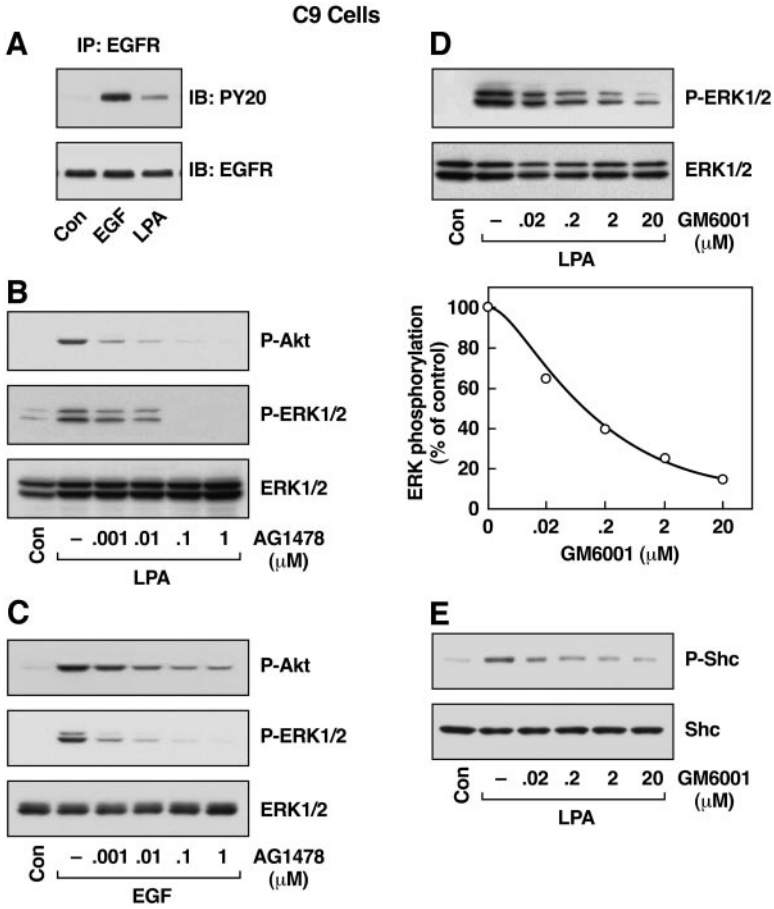

Fig. 8. MMP Dependence of LPA-Induced EGF-R Transactivation and ERK1/2 Phosphorylation in Hepatic C9 Cells

A, LPA and EGF stimulate tyrosine phosphorylation of the EGF-R. After stimulation with LPA (10 μM) and EGF (20 ng/ml) for 2 min, cell lysates were immunoprecipitated (IP) with anti-EGF-R antibody and immunoblotted (IB) with phosphotyrosine antibody (PY20). B and C, Concentration-dependent inhibition by AG1478 of Akt and ERK1/2 activation by LPA and EGF. D and E, Effects of metalloproteinase inhibition by GM6001 on LPA-induced phosphorylation of ERK1/2 and Shc. C9 cells were treated with increasing concentrations of GM6001 for 30 min and stimulated with LPA (10 μM) for 5 min. Con, Control; P-Akt, phosphorylated Akt; P-Shc, phosphorylated Shc.

Nonreceptor protein tyrosine kinases such as Src and Pyk2 are important intermediates of signal transduction cascades, controlling pathways as diverse as cell growth, differentiation, migration, secretion, and genome maintenance (56). These kinases are also involved in regulation of the cytoskeleton due to their ability to associate with various cytoskeletal proteins, including focal adhesion kinase. Pyk2 belongs to the focal adhesion kinase family and is involved in cell proliferation and migration (57, 58). Src and Pyk2 are activated by GPCRs, integrins, cytokines, and growth factors and have been shown to be indispensable for EGF-R activation (20, 59, 60). Our data in BAG cells indicate that LPA causes rapid Src-dependent phosphorylation of Pyk2 at Tyr402, and that inhibition of Src attenuates EGF-R activation and ERK1/2 phosphorylation (Figs. 2 and 5). An important role of Src has been observed in the modulation of aldosterone secretion. The Src inhibitor, PP2, has been shown to inhibit basal as well as agonist-mediated aldosterone production in response to stimulation with angiotensin II, K^+, and dibutyryl-cAMP in adrenocortical H295R cells, possibly through inhibition of early steps in steroidogenesis (3, 60). Because Pyk2 is activated by Src, it would be of interest to examine its role in agonist-induced steroidogenesis in BAG cells.

Activation of the EGF-R and PI3K by GPCR agonists has been implicated in cell proliferation, migration, and invasion (36, 61). To evaluate the impact of LPA signaling on the growth of these cells, we examined the role of PI3K/Akt in these cells. This revealed that agonist-induced ERK1/2 activation was reduced by inhibition of PI3K, and that LPA causes activation of Akt in a PI3K-dependent manner (Fig. 10). LPA-induced activation of PI3K/Akt was mediated by the EGF-R, but without any effect on ERK1/2 phosphorylation in C9 cells. However, in BAG cells, PI3K inhibition impairs ERK1/2 activation by LPA. Whereas inhibition of Src decreased LPA responses to basal levels, PI3K inhibition markedly reduced proliferation to below the

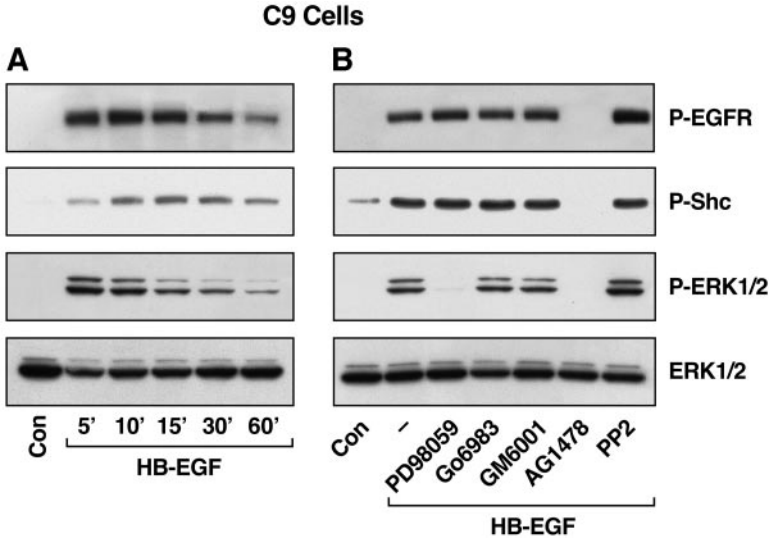

Fig. 9. HB-EGF Stimulates Phosphorylation of the EGF-R and Activation of ERK1/2 in C9 Cells

A, Time course of the effects of HB-EGF (20 ng/ml) on phosphorylation of the EGF-R (Tyr1173), Shc, and ERK1/2 in C9 cells. B, Effects of inhibition of MEK1/2 by PD98059 (10 μM), PKC by Go6983 (1 μM), metalloproteinase by GM6001 (20 μM), EGF-R by AG1478 (200 nM), and Src by PP2 (5 μM) on phosphorylation of the EGF-R (Y1173), Shc, and ERK1/2 by HB-EGF. C9 cells were treated with the inhibitors for 15 min and stimulated with HB-EGF (20 ng/ml) for 5 min. Con, Control; P-EGFR, phosphorylated EGF-R; P-Shc, phosphorylated Shc.

basal level in both cell types (Fig. 10). In summary, the present findings indicate that LPA-induced EGF-R activation and mitogenic signaling in cultured BAG cells is independent of MMP induction. Whereas Src activation is essential for LPA-induced phosphorylation of ERK1/2 and cell proliferation, PI3K is necessary for the maintenance of normal cell survival. Moreover, whereas LPA-induced activation of ERK1/2 and RSK-1 occurs through dual signaling cascades involving activation of PKC and EGF-R in a MMP-independent manner in BAG cells, it is solely mediated by G_i through MMP-dependent transactivation of the EGF-R in C9 cells (Fig. 11).

MATERIALS AND METHODS

DMEM, Medium 199, donor horse serum, fetal bovine serum, and antibiotic solutions were from GIBCO Invitrogen Corp. (Carlsbad, CA). PKC inhibitors, PP2, wortmannin, CRM197, AG1478, GM6001, pertussis toxin, phospho-EGF receptor (Y845 and Y1173), phospho-Pyk2 (Y402) and anti-Shc antibodies were purchased from Calbiochem (La Jolla, CA); human recombinant EGF was from Invitrogen or Biosource International (Camarillo, TX). Recombinant HB-EGF and anti-HB-EGF antibody were from R & D Systems, Inc. (Minneapolis, MN). Dominant negative EGF-R plasmid was provided by Dr. Yosef Yarden and PKC isoform mutants were supplied by Dr. J.-W. Soh (Columbia University, New York, NY) (62); C-terminal Src kinase was provided by Dr. Zvi Naor (Tel Aviv University, Tel Aviv, Israel). Dominant negative H-Ras (S17N) and anti-phospho-Src (Y416) antibody were from Upstate Biotechnology (Lake Placid, NY). Antibodies to phospho-RSK-1, RSK-1, actin, EGF-R, and Src were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), or Cell Signaling Technology (Beverly, MA). Phosphotyrosine (PY20) and Pyk2 antibodies were from Transduction Laboratories, Inc. (Lexington, KY) or Cell Signaling Technology, Inc. Anti-phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 antibodies were from Cell Signaling Technology, Inc. Secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard and Perry Laboratories (Gaithersburg, MD), and enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Arlington Heights, IL) or Pierce Chemical Co. (Rockford, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Primary cultures of adrenal glomerulosa cells were prepared from bovine adrenal glands as previously described (63). Cells were plated in six-well plates in DMEM containing 10% (vol/vol) donor horse serum, 2% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, 5 μg/ml fungizone, and 25 μg/ml gentamycin. Clone 9 (C9) rat liver epithelial cells were grown in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% (vol/vol) fetal calf serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 250 μg/ml fungizone. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 C for 2–3 d, after which time they formed confluent monolayers and were rendered quiescent by withdrawal of serum for 24 h before use. After stimulation for the time periods indicated in the individual experiments, cells were washed with ice-cold PBS, lysed with Laemmli buffer, and frozen at −70 C before analysis.

Cell Transfections

Cells were transfected with plasmid DNA using Neucleofector (Amaxa Biosystems, Gaithersburg, MD), which allows nonviral gene transfer directly into the nucleus using specific Nucleofector solutions and applying specific electrical pa-

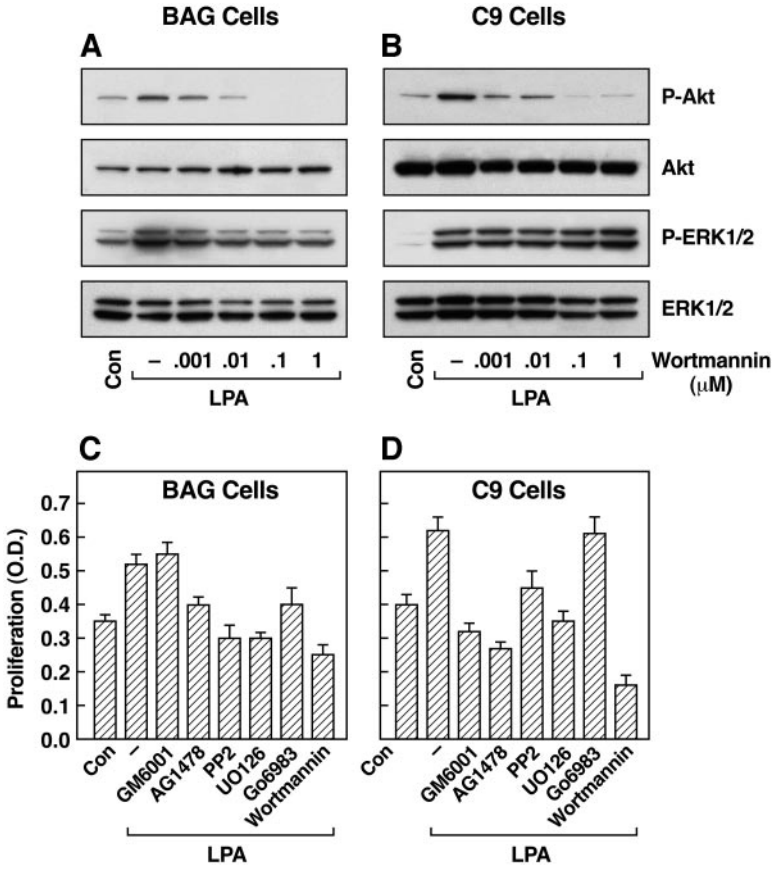

Fig. 10. Role of PI3K in Agonist-Induced Phosphorylation of Akt and ERK1/2 in BAG and C9 Cells

A and B, Concentration-dependent effects of PI3K inhibition by wortmannin on phosphorylation of Akt at Ser473 and ERK1/2 by LPA. Cells were treated with increasing concentrations of wortmannin and stimulated with LPA (10 μM) for 5 min and then lysed in Laemmli sample buffer and analyzed by SDS-PAGE. C and D, Mechanism of LPA-induced cell proliferation. Cells grown in 96-well plates were treated with the Src inhibitor, PP2 (10 μM), EGF-R antagonist, AG1478 (1 μM), PI3K inhibitor, wortmannin (100 nM), MEK inhibitor, U0126 (1 μM), PKC inhibitor Go6983 (1 μM), and MMP inhibitor, GM6001 (20 μM), for 30 min followed by addition of LPA (10 μM). Cell proliferation was measured as described in *Materials and Methods*. Con, Control; P-Akt, phosphorylated Akt.

rameters optimized for epithelial cells. Briefly, cells were grown in 75-cm flasks, washed with sterile HEPES-buffered saline, and collected after trypsinization in DMEM. Cell suspension was centrifuged at 1000 rpm for 10 min and supernatant was discarded. The cell pellet was resuspended in Basic Nucleofector Solution (Amaxa Biosystems) to a final concentration of 10^6 cells/100 μl and mixed with 1 μg of DNA. The nucleofection sample was transferred to cuvette and run in the nucelofector using an appropriate program. After transfection, cells were transferred to six-well plates for 2 d and incubated in serum-free medium for 16 h before experimental treatments.

Immunoprecipitation

After treatment with inhibitors and drugs, cells were placed on ice and washed twice with ice-cold PBS, and then lysed in RIPA lysis buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 20 mM NaF, 10 mM Na-pyrophosphate, 5 mM EDTA, 1% Nonidet P-40 (NP-40), 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin, and 1 mM 4-(2-aminoethyl)benzensulfonyl fluoride, and probe-sonicated (Sonifier Cell Disruptor). Solubilized lysates were clarified by centrifugation at 8000 × *g* for 10 min, precleared with agarose, and then incubated with specific antibodies and protein A or G agarose. The immunoprecipitates were collected, washed four times with lysis buffer, and dissolved in Laemmli buffer. After heating at 95 C for 5 min, the samples were centrifuged briefly, and the supernatants were analyzed by SDS-PAGE on 8–16% gradient gels.

Immunoblot Analysis

Cells were grown in six-well plates and at 60–70% confluence were serum starved for 24 h before treatment at 37 C with selected agents. The media were then aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 μl Laemmli sample buffer. The samples were briefly sonicated, heated at 95 C for 5 min, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8–16%) gradient gels and transferred to polyvinylidine difluoride membranes. Blots were incubated overnight at 4 C with primary antibodies and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with enhanced chemiluminescence reagent (Pierce Chemical Co.) and quantitated with a scanning laser densi-

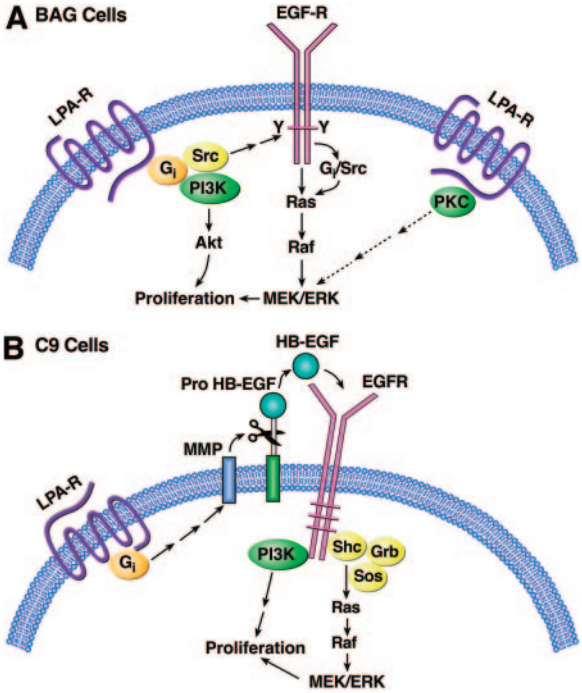

Fig. 11. Differential LPA- and EGF-Stimulated Signaling Pathways during ERK1/2 Activation in BAG and C9 Cells

A, LPA stimulation of BAG cells causes activation of G_i, Src, and EGF-R, leading to activation of the Ras/MEK/ERK cascade. PKC has a partial role in LPA action and appears to act downstream of Ras. EGF stimulation is dependent on activation of G_i and Src, but is independent of PKC. Agonist-induced activation of PI3K makes a greater contribution to cell proliferation than ERK1/2 phosphorylation in BAG cells. B, In C9 cells, LPA-induced ERK1/2 phosphorylation is primarily mediated through activation of G_i and MMP-dependent generation of HB-EGF, which binds to and activates the EGF-R. This leads to recruitment of the adaptor molecules, Shc, Grb, and Sos, and activation of the Ras/Raf/MEK/ERK cascade. However, PKC has no role in LPA signaling in C9 cells. LPA-R, LPA receptor.

tometer. In some cases, blots were stripped and reprobed with other antibodies.

Ras Activation Assay

The active form of Ras was determined using the EZ-Detect Ras Activation Kit (Pierce Chemical Co.). The active GTP-bound Ras was extracted from cell lysates with the glutathione-*S*-transferase/Raf1/Ras-binding domain, and the pull-down active Ras was detected by Western blot analysis using anti-Ras antibody.

Cell Proliferation Assay

Measurement of cell proliferation was done by addition of CellTiter 96^R AQ_{ueous} One Solution Reagent (Promega Corp., Madison, WI), which contains a tetrazolium compound (MTS-tetrazolium) and an electron-coupling reagent (phenazine ethosulfate). Briefly, cells were grown in 96-well microtiter plates and changed to serum-free media at 50–60% confluence. Inhibitors were added 30 min before the addition of agonists and left for 24 h. The CellTiter 96^R AQ_{ueous} reagent (20 μl) was added, and plates were kept in a humidified incubator at 37 C for 2–3 h, after which absorbance was measured at 490 nm.

Acknowledgments

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